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(Reaffirmed 2005)

भारतीय मानक

खाद्य विषाक्तता उत्पन्न करने वाले जीवाणुओं के संसूचन की पद्धतियाँ

भाग 4 *क्लौसट्रीडियम परफ्रिंजेंस (क्लौसट्रीडियम वेलशी)* और *क्लौसट्रीडियम बोटूलिनम* का विलगन और पहचान तथा *क्लौसट्रीडियम परफ्रिंजेंस* का गणन

(दूसरा पुनरीक्षण)

Indian Standard

METHODS FOR DETECTION OF BACTERIA RESPONSIBLE FOR FOOD POISONING

PART 4 ISOLATION AND IDENTIFICATION OF *CLOSTRIDIUM PERFRINGENS* (*CLOSTRIDIUM WELCHII*) AND *CLOSTRIDIUM BOTULINUM* AND ENUMERATION OF *CLOSTRIDIUM PERFRINGENS*

(Second Revision)

ICS 07.100.30

BUREAU OF INDIAN STANDARDS
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June 1999

Price Group 2

FOREWORD

This Indian Standard (Part 4) (Second Revision) was adopted by the Bureau of Indian Standards, after the draft finalized by the Food Microbiology Sectional Committee had been approved by the Food and Agriculture Division Council.

Several micro-organisms contaminating food give rise to clinical symptoms, such as abdominal pain, nausea, vomitting, diarrhoea and sometimes pyrexia. A well-known exception is that of botulism where the symptoms are those of difficulty in swallowing, diplopia, aphonia and difficulty in respiration. Poisoning through food is characterized by symptoms of explosive nature which occur in otherwise healthy individuals. Such explosive nature of food poisoning helps in differentiating conditions from those of out-breaks of food-borne infectious diseases which generally spread over a period of several days. This part of the standard covers the method for isolation and identification of some clostridium species responsible for food poisoning.

This standard was published in 1970 and was first revised in 1976.

This standard (Part 4) is being revised in order to cover the method for *Bacillus cereus* in a separate part (Part 6) and to align with ISO 7932 : 1993 'Microbiology — General guidance for the enumeration of *Bacillus cereus* — Colony-count technique at 30°C.'

In reporting the result of a test or analysis made in accordance with this standard, if the final value, observed or calculated, is to be rounded off, it shall be done in accordance with IS 2 : 1960 'Rules for rounding off numerical values (*revised*)'.

Indian Standard

METHODS FOR DETECTION OF BACTERIA RESPONSIBLE FOR FOOD POISONING

PART 4 ISOLATION AND IDENTIFICATION OF *CLOSTRIDIUM PERFRINGENS* (*CLOSTRIDIUM WELCHII*) AND *CLOSTRIDIUM BOTULINUM* AND ENUMERATION OF *CLOSTRIDIUM PERFRINGENS*

(Second Revision)

1 SCOPE

This standard (Part 4) prescribes methods for isolation and identification of *Clostridium perfringens* (*Clostridium welchii*) and *Clostridium botulinum* and enumeration of *Clostridium perfringens* in foods.

2 REFERENCES

The following Indian Standards contain provisions which through reference in this text, constitute provision of this standard. At the time of publication, the editions indicated were valid. All standards are subject to revision and parties to agreements based on this standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below:

IS No.	*Title*
1070 : 1992	Reagent grade water (*third revision*)
5404 : 1984	Code of practice for handling of samples for microbiological analysis (*first revision*)
6850 : 1973	Agar, microbiological grade
6851 : 1973	Meat extract, microbiological grade
6853 : 1973	Peptone, microbiological grade
7004 : 1973	Yeast extract, microbiological grade
7128 : 1973	Proteose peptone, microbiological grade
7536 : 1975	Soluble starch, microbiological grade
10232 : 1982	Guidelines for preparation of dilutions for microbiological examination of food

3 SAMPLING

For microbiological examination the samples should be handled carefully. For this purpose, IS 5404 shall be followed.

4 QUALITY OF REAGENTS

Unless specified otherwise, pure chemicals shall be employed in tests and reagent grade water (*see* IS 1070) shall be used where use of water as a reagent is intended.

NOTE — 'Pure chemicals' shall mean chemicals that do not contain impurities which affect the test results.

5 GENERAL CHARACTERISTICS

5.1 *Clostridium perfringens (Cl. welchii)*

Anaerobic, Gram-positive rods, about 4 to 6 μ × 1 μ with square ends. Spores are formed when grown in Ellner's medium (**6.6**), and are oval, subterminal and not bulging. Typical food-poisoning strains of *Clostridium perfringens* are non-haemolytic on horse blood agar (**6.3**), and produce lecithinase as demonstrable on the egg-yolk plates (**6.5**) in showing precipitates around the colonies. The spores are heat resistant at 100°C for one hour; however, some workers have reported reduced heat resistance of the spores when cultures are grown in Ellner's medium (**6.6**). The classification of *Clostridium perfringens* into various types have been made on the basis of 'toxins'. The three types of *Clostridium perfringens* commonly associated in food poisoning are Types A, C and D.

5.1.1 In practice, the method of identification of food-poisoning strains of *Clostridium perfringens* are based on colonial characters, morphology and the Nagler reaction. It should be noted that the frequency of spore-bearing *Clostridium perfringens* is low and reduces the diagnostic values of this criterion.

5.2 *Clostridium botulinum*

Anaerobic, Gram-positive rods, large and stout with straight sides and rounded ends. Spores are oval, central or subterminal and distend the bacillary body. On blood agar medium (**6.3**), growth is associated with haemolysis which may not be larger than the

colony. On egg-yolk medium (**6.5**), colonies produce opalescence and a pearly layer and are lactose negative.

5.2.1 Guinea pigs inoculated intra-peritoneally with botulinum toxin show difficulty in breathing, flaccid paralysis of the abdominal muscles and salivation. At autopsy, marked congestion of internal organs, extensive thrombosis and haemorrhages are noted. The toxic activity may be neutralized by botulinum antitoxin, and the toxin is thermolabile.

5.2.2 Toxin of *Cl. botulinum* Type E is also thermolabile, is activated by trypsin and may conveniently be demonstrated using white mice.

6 MEDIA

6.1 Nutrient Broth

Mix and dissolve by heating 10 g peptone (*see* IS 6853), 10 g meat extract (*see* IS 6851), 5 g sodium chloride in 1 000 ml water. When cool, adjust *p*H from 7.5 to 7.6. Remove precipitate by filtration through filter paper. Sterilize by autoclaving at 120°C for 15 min.

6.2 Nutrient Agar

To the medium as in **6.1** add agar (*see* IS 6850) in such a concentration as to solidify and produce a sufficiently firm surface when poured in sterile petri dishes. The concentration of agar to be added varies from batch to batch and needs to be adjusted accordingly. Usual concentrations required vary from 1.5 to 3.0 percent. Dissolve the agar in the nutrient broth and sterilize by autoclaving at 120°C for 15 min. Plates and slopes are prepared from sterile nutrient agar.

6.3 Blood Agar with Neomycin

Melt sterile nutrient agar (**6.2**) and hold between 50 to 55°C in a water-bath. Add sterile blood free from preservatives to give a concentration of 10 percent. Mix well and pour plates. Horse blood is commonly used but when not available, that of sheep, human or rabbit may be used. Add aseptically neomycin sulphate, 70 µg per ml, before pouring in plates.

6.4 Cooked Meat Medium

Mince 500 g fresh beef heart and place in 500 ml alkaline boiling water containing 1.5 ml 1 N sodium hydroxide solution. Simmer for 20 min. Drain off the liquid through muslin filter while still hot and partially dry the meat in the cloth or on filter papers. To 500 ml of the liquid filtered from the cooked meat add 2.5 g peptone (*see* IS 6853) and 1.25 g sodium chloride. Steam at 100°C for 20 min and add 1 ml concentrated hydrochloric acid and filter. Bring the reaction of the filtrate to *p*H 8.2 and steam again at 100°C for 30 min; adjust to *p*H 7.8. Place meat in test-tubes or in about 30 ml screw-capped bottles to a depth of about 2.5 cm and cover with 10 ml of the broth obtained. Autoclave at 120°C for 20 min. A layer of sterile paraffin may be added to cover the surface, if necessary.

6.5 Willis and Hobb's Medium with Neomycin

Break fresh eggs which have been scrubbed and then sterilized by immersing in 0.1 percent aqueous solution of mercuric chloride with precaution to keep the contents sterile and separate the yolk from the white. Discard the egg white and suspend yolk in equal volume of sterile 0.9 percent solution of sodium chloride. Prepare separately sterile milk by autoclaving skimmed milk at 120°C for 15 min. Skimmed milk is prepared by centrifuging ordinary milk to remove the cream. Dissolve by steaming, 4 to 5 g agar (*see* IS 6850) and 4.8 g lactose in 400 ml of nutrient broth (**6.1**) of *p*H 7.0 to which has been added 1 percent solution of neutral red, 1.3 ml. Sterilize at 120°C for 15 min. Cool to 50 to 55°C and add 15 ml egg yolk solution, 60 ml sterile skimmed milk and sterile solution of neomycin sulphate to a final concentration of 70 µg per ml.

6.6 Spore Inducing Medium (Ellner's Medium)

Dissolve salts and ingredients separately in 1 000 ml water by brief steaming at 100°C, 10 g proteose peptone (*see* IS 7128), 3 g yeast extract (*see* IS 7004), 3 g soluble starch (*see* IS 7536), 0.1 g magnesium sulphate ($MgSO_4$), 1.5 g potassium dihydrogen phosphate (KH_2PO_4), and 50 g disodium hydrogen phosphate ($Na_2HPO_4,7H_2O$). Adjust to *p*H 7.8. After dispensing in test tubes of 150 mm × 20 mm to two-thirds full, sterilize by autoclaving at 120°C for 15 min.

6.7 Medium for *Cl. Botulinum* Type E

To the ingredients as in cooked meat medium (**6.4**), add 5 percent trypticase, and 0.5 percent peptone (*see* IS 6853). Adjust *p*H to 7.0. Sterilize at 120°C for 20 minutes and add a sterilized solution of 10 percent sodium thioglycollate and 20 percent glucose to have final concentrations of 0.2 and 0.4 percent respectively.

7 PROCEDURE FOR ISOLATION

7.1 *Clostridium perfringens*

Where necessary, the sample is blended in a sterile blender/jar for 2 minutes or macerated with sterile sand in a sterile mortar using approximately 200 ml of diluting fluid per approximately 25 g of the sample. The diluting fluid should be 0.1 percent peptone (*see* IS 6853) in water sterilized at 120°C for 20 min, final *p*H 6.8 ± 0.1 *or* 3.4 percent of potassium

dihydrogen phosphate (KH_2PO_4) in water, *p*H adjusted to 7.2 and sterilized at 120°C for 20 min. An aliquot of the specimen is inoculated into cooked meat medium (**6.4**) and the inoculated tube heated in a steamer at 100°C for one hour and incubated overnight at 37°C. An aliquot of the specimen is also inoculated directly on to blood agar medium (**6.3**) and the egg-yolk medium (**6.5**) and incubated in an anaerobic jar at 37°C overnight. Subcultures are made from the growth in medium (**6.4**) on to the two solid media (**6.3** and **6.5**) and incubated in an anaerobic jar at 37°C overnight.

7.2 ***Cl. botulinum***

Preheat the sample at 80°C for 30 min and inoculate into cooked meat medium (**6.4**), and the two solid media, **6.3** and **6.5**. The solid media are incubated anaerobically, and all the three inoculated media are incubated at 37°C for 5 to 10 days.

The type E strains exhibit low thermal resistance, and are missed in specimens which have been heated prior to inoculation. For the isolation of these strains, the procedure as in **7.2.1** should be followed.

7.2.1 *Isolation of Cl. Botulinum Type E*

Inoculate the specimen in duplicate tubes of the medium (**6.7**), and incubate at 30°C for 3 days. In sterile test tubes take aliquots of 2 ml samples of growth and mix with equal volume of absolute ethanol. Let stand at 25°C for one hour with occasional mixing. Streak on to egg-yolk medium (**6.5**) and inoculate into medium (**6.7**). Incubate overnight at 37°C, the solid medium being incubated in an anaerobic jar. Examine the solid medium for presence of colonies with opalescence zones indicating growth of *Cl. botulinum* Type E. If such colonies are present, carry out test for toxin using the growth in medium (**6.7**) inoculated with ethanol treated culture.

8 TESTS FOR IDENTIFICATION

8.1 ***Clostridium perfringens***

8.1.1 *Gram's Stain*

The stain consists of:

a) 0.5 percent methyl violet or crystal violet in water,

b) iodine solution (1 percent iodine and 2 percent potassium iodide in water), and

c) counterstain (0.1 g neutral red, 0.2 ml of 1 percent acetic acid and 100 ml water).

On a clean grease-free slide, very light and thin smear covering a small area is made directly from liquid culture and in clean tap water if from solid media. The smear is fixed by passing to and fro over a flame and cooled. Cover the smear with the stain (a) for 30 s, pour off the stain and wash with (b) and then cover with (b) and allow to remain for 30 s. Wash off with ethanol until the dye ceases to stream out. Wash in running tap water and apply (c) for about one minute. Wash in tap water and dry for examination.

8.1.2 *Colonial Characters*

By growth on blood agar medium (**6.3**) and egg-yolk medium (**6.5**), as described in (**5.1**).

8.1.3 *Spore Production*

Inoculate growth from any of the media as in **6.3** and **6.5** into spore inducing medium (**6.6**). The growth in cooked meat medium (**6.4**) after following the procedure as in **7.1** may also be inoculated into medium (**6.6**).

NOTE — Some workers have noted reduced heat resistance of spores when cultures are grown in **6.6**.

8.1.4 *Nagler Reaction — In Vitro Test for Alpha Toxin*

One half of a plate of egg-yolk medium (**6.5**) is spread over with two or three drops of standard *Clostridium perfringens* antitoxin and dried. The area is demarcated. The two halves of the plate are inoculated with the suspect strain and incubated at 37°C anaerobically. Lecithinase activity is shown by precipitates around colonies in the half without antitoxin and this reaction is inhibited in the other half with specific antitoxin. The production of the enzyme lecithinase C, as demonstrated in the Nagler reaction, by all types of *Clostridium perfringens* is used to distinguish *Clostridium perfringens* from other species of *Clostridia*. However, *Cl. bifermentes* also produce lecithinase and may be differentiated from *Clostridium perfringens* by *Cl. bifermentes* showing proteolytic activity, ready sporulation and non-fermentation of lactose. Lactose fermentation is carried out in 1 percent peptone water sugar medium incubated anaerobically at 37°C.

8.2 ***Cl. botulinum***

8.2.1 *Gram's Stain*

See **8.1.1**.

8.2.2 *Colonial Characters*

By growth on blood agar medium (**6.3**) and egg-yolk medium (**6.5**), as described in **5.2**, and for Type E strains as in **7.2.1**.

8.2.3 *In Vivo Test for Toxin*

Grow suspect strain in cooked meat medium (**6.4**) for 5 to 10 days. Obtain filtrate and divide into two

portions, one of which is heated at 100°C for 10 min. Use three guinea pigs for intraperitonal injection with filtrate as follows:

a) One animal is protected with polyvalent botulinum antitoxin and injected with 2 ml of unheated filtrate;

b) One animal as injected with 2 ml of unheated filtrate and is unprotected; and

c) One animal is injected with 2 ml of heated filtrate.

Death with paralytic symptoms of the unprotected animal receiving unheated filtrate and survival of the other two animals diagnose the presence of botulinum toxin.

8.2.4 *Demonstration of Toxin of Cl. Botulinum Type E*

The procedure as in **8.2.3** may fail to demonstrate toxin of *Cl. botulinum* Type E. For such strains the procedure shall be as follows.

8.2.4.1 To filtrate from growth in medium (**6.7**) as obtained after procedure described in **7.2.1** is added trypsin to a final concentration of 0.1 percent. Incubate at 37°C for 60 min. Dilute specific Type E antitoxin 1 in 5 with 0.1 M phosphate buffer of *p*H 6.5 containing 0.2 percent gelatin. To 1.5 ml of diluted antitoxin, add equal volume of trypsinized filtrate, mix and keep at room temperature for 30 min. Inject 1 ml of the mixture intra-peritoneally into a pair of white mice. Also inject a pair of mice with 0.5 ml of the filtrate heated at 100°C for 10 min and another pair of mice with 0.5 ml of unheated trypsinized filtrate. Observe the mice up to 96 h. Death of the unprotected mice and survival of the mice receiving neutralized toxin and the heated toxin diagnose toxin of *Cl. botulinum* Type E.

8.2.5 Since bacteriological diagnosis of food-poisoning due to *Cl. botulinum* is based on the demonstration of the toxin in the food or intestinal content, the presence of the toxin in such materials need to be demonstrated.

8.2.5.1 *Procedure*

The material is soaked overnight in equal volume of sterile normal saline. The suspension is centrifuged and the supernatant sterilized by filtration. This is then directly used to note the presence of *botulinum* toxin by animal inoculation as with culture filtrate described in **8.2.3** and **8.2.4**.

9 SEROTYPING

Food poisoning strains of *Clostridium perfringens* may be serotyped by slide agglutination using colonies from blood agar (**6.3**), and testing with specific agglutinating sera, if these are available.

10 ENUMERATION

10.1 ***Clostridium perfringens***

25 to 50 g of the sample is taken in a sterile blender/jar and to this is added diluting fluid (*see* **7.1**) to have dilution of 10^{-1}. Blend at 8 000 to 10 000 rev/min for 2 min. Alternatively, macerate the sample with diluting fluid in a sterile mortar with sterile sand. Make serial ten-fold dilutions with the diluting fluid in duplicate series up to 10^{-7}. Streak 0.1 ml from each tube evenly on to blood agar medium (**6.3**) and also on to egg-yolk medium (**6.5**). Incubate in an anaerobic jar at 37°C for 18 to 24 h. It is useful to incubate aerobically duplicate plates similarly inoculated for comparison. The suspect colonies (**5.1**) are counted and the number of viable colonies per gram of sample determined by multiplying by the dilution factor(s) and dividing by the mass of the sample.

10.1.1 Since food-poisoning occurs due to the presence of large number of *Clostridium perfringens*, usually millions per gram; therefore, counts of colonies shall be of considerable help particularly in surveys for clostridial invasion of food producing factories.

Bureau of Indian Standards

BIS is a statutory institution established under the *Bureau of Indian Standards Act*, 1986 to promote harmonious development of the activities of standardization, marking and quality certification of goods and attending to connected matters in the country.

Review of Indian Standards

Amendments are issued to standards as the need arises on the basis of comments. Standards are also reviewed periodically; a standard along with amendments is reaffirmed when such review indicates that no changes are needed; if the review indicates that changes are needed, it is taken up for revision. Users of Indian Standards should ascertain that they are in possession of the latest amendments or edition by referring to the latest issue of 'BIS Handbook' and 'Standards : Monthly Additions'.

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